et al., 1992). Therefore, it is unlikely that an increase in cyclic AMP is involved in the inhibitory effect of azelastine on the Ca^{2+} current. Also, the inhibitory effect of azelastine on K^+ currents does not appear to be mediated by cyclic AMP, because in tracheal smooth muscle cells, isoproterenol, which increases cyclic AMP, activates Ca^{2+}-dependent K^+ currents (Kume et al., 1989).

Recent papers showed that azelastine also inhibits agonist-induced Ca^{2+} release and agonist-induced Ca^{2+} sensitization of contractile elements in guinea-pig ileal and tracheal smooth muscle cells (Masuo et al., 1992; Sanagi et al., 1992). In the present study, we could not determine the mechanism by which azelastine produces bronchodilator activity. However, the potent effect of azelastine to inhibit I_{Ca} as well as the above-mentioned actions makes it a very promising drug to relax airway smooth muscle.

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Biological activities of two endogenously occurring *N*-terminally extended forms of galanin in the rat spinal cord

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Abstract

The occurrence of two *N*-terminally extended forms of galanin in the porcine adrenal medulla was reported earlier by Bersani et al. (1991). We have synthesized and examined the ability of these two extended forms of galanin, galanin-(−7–29) and galanin-(−9–29), to bind to galanin receptors in the rat dorsal spinal cord. The effect of intrathecal (i.t.) injection of these peptides on spinal flexor reflex excitability in decerebrate, spinalized, unanesthetized rats was also studied. Both galanin-(−7–29) and galanin-(−9–29) fully displaced specific ^{125}I-monoiodo-[Tyr^{26}]porcine galanin (^{125}I-galanin) binding to membranes prepared from rat dorsal spinal cord, with IC_{50} values 0.13 and 0.14 μM, respectively. The metabolic half-lives in spinal cord membranes for galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) were 117 ± 17, 271 ± 23 and 185 ± 19 min, respectively. I.t. injection of galanin-(−7–29) and galanin-(−9–29) mimicked the biphasic facilitatory and inhibitory effect of i.t. galanin-(1–29) on flexor reflex excitability and antagonized C-fiber conditioning stimulus-induced spinal cord hyperexcitability, but with reduced potencies compared to galanin-(1–29). We suggest that the *N*-terminally extended forms of galanin act as endogenous ligands with low agonist activity.

Key words: Preprogalanin; Galanin; Galanin receptor; Nociception; Spinal cord; Neuropeptide

1. Introduction

Galanin, a neuropeptide of 29 or 30 (human) amino acids was originally isolated from porcine upper intestine (Tatemoto et al., 1983). The precursor, preprogalanin, a 123-amino-acid-long protein, containing a leader sequence: galanin and a flanking sequence: galanin message-associated peptide, is processed to yield galanin and galanin message-associated peptide in a stochiometric ratio (Rökaeus and Carlquist, 1988). Galanin and galanin message-associated peptide-like immunoreactivites are widely distributed in the peripheral and central nervous systems, with partly overlapping and differential distributions, possibly due to a tissue-specific, post-translational alternative processing of preprogalanin (Ch'ng et al., 1985; Hökfelt et al., 1992; Melander et al., 1986; Skofitsch and Jacobowitz, 1985). Receptor autoradiographic studies, using mono-[^{125}I]Tyr^{26}-porcine galanin (^{125}I-galanin) as radioligand, have shown a distribution of galanin receptors similar to that of the galanin-like immunoreactivity, with a high concentration of binding sites in the superficial layers of the rat lumbar dorsal spinal cord (Melander et al., 1988; Skofitsch et al., 1986). In the spinal cord, intrathecal (i.t.) galanin-(1–29) has a biphasic facilitatory and inhibitory effect on the flexor reflex and dose dependently inhibits the prolonged sensitisation of spinal cord excitability induced by repetitive C-fiber stimulation (Wiesenfeld-Hallin et al., 1989; Xu et al., 1990). See Wiesenfeld-Hallin et al. (1992) for review.

Recently, several groups have demonstrated molecular heterogeneity of galanin-like immunoreactivity in different species and tissues. Both truncated and extended forms of galanin-(1–29) were found by molecu-

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lar analysis of galanin-like immunoreactive material (Bauer et al., 1986a,b; Bersani et al., 1991a,b; McDonald et al., 1992; Michener et al., 1990; Sillard et al., 1992). Two *N*-terminally extended forms of galanin, galanin-(−7–29) and galanin-(−9–29), were found in substantial amounts in, and were released from, the porcine adrenal medulla (Bersani et al., 1991b). The biological activity of these peptides as putative endogenous galanin receptor ligands has, however, not yet been reported upon. To determine whether or not the *N*-terminally extended forms of galanin could contribute to galaninergic actions at the spinal galanin receptors, we have synthesised galanin-(−7–29) and galanin-(−9–29) and studied their ligand binding properties and biological actions in the depression of spinal cord excitability induced by C-fiber conditioning stimulation.

2. Materials and methods

2.1. Materials

$Na^{125}I$ (2500 Ci/mmol) was purchased from Amersham. The different galanin receptor ligands were synthesized, purified and characterized as described by Langel et al. (1992). Galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) were all of the porcine galanin sequence. Mono-[^{125}I]-Tyr^{26}-galanin was prepared by chloramine-T iodination of porcine galanin as described by Land et al. (1991b). Briefly, 10 μg porcine galanin-(1–29) was iodinated using a 4-fold excess of peptide over $Na^{125}I$, to yield mono-[^{125}I]Tyr^{26}-porcine galanin (ca. 1000 Ci/mmol). All other reagents were from Sigma.

2.2. Preparation of membranes from lumbar dorsal spinal cord

Adult male rats (Sprague-Dawley 180–200 g) were decapitated, the lumbar spinal cord was rapidly dissected and divided into dorsal and ventral parts. The tissue (10% w/v) was homogenized on ice with a tight fitting teflon-glass homogenizer (10 strokes at 695 rpm) in 0.32 M sucrose buffered with 5 mM Hepes (pH 7.4). The homogenate was diluted 10-fold with sucrose and centrifuged at 1000 × *g* for 10 min. The supernatant was further centrifuged at 10000 × *g* for 45 min and the pellet was resuspended in 5 mM Hepes-buffered Krebs-Ringer solution (Hepes-KR), containing 137 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, 2.05 mM $MgCl_2$, 1 g l^{-1} glucose, pH 7.4.

2.3. Equilibrium binding studies

Displacement experiments were performed in a final volume of 400 μl 5 mM Hepes-KR solution, supplemented with 0.05% (w/v) bovine serum albumin and 1 mg/ml bacitracin, containing 0.2 nM porcine ^{125}I-galanin, 70–100 μg of the lumbar dorsal spinal cord membrane preparation (P_2) and varying concentrations of unlabeled galanin, galanin-(−7–29) or galanin-(−9–29) (10^{-11} to 10^{-4} M). Samples were incubated for 30 min at 37°C. The incubation was terminated by the addition of 2 × 10 ml of ice-cold 5 mM Hepes-KR, supplemented with 0.05% (w/v) bovine serum albumin, followed by rapid filtration over Whatman GF/C filters precoated 2–3 h in 0.3% (v/v) polyethylenimine (mw 50 kD) solution. Specific binding was defined as that displaceable by 1 μM galanin. Where indicated, a protease inhibitor cocktail consisting of (mg/ml): antipain-papain and trypsin inhibitor (0.1), bestatin-aminopeptidase inhibitor (0.08), chymostatin (0.2), E-64-cystein protease inhibitor (0.5), leupeptin-serine and cysteine protease inhibitor (0.001), pepstatin-aspartic protease inhibitor (0.0015), EDTA(1) phosphoramidon-metallo-endoprotease inhibitor (0.32), APMSF-serine protease inhibitor (0.08) and aprotinin-serine protease inhibitor (0.02) (all from Boehringer Mannheim) was used. All tubes and tips used for peptide stock solutions were silanized with Sigmacote prior to the experiments.

2.4. Degradation experiments

To determine the rate of degradation of galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) (20 μM), the peptides were incubated with P_2-membranes (0.2 mg/ml) from lumbar dorsal spinal cord at 37°C, 0–210 min. The degradation was stopped and proteins were precipitated by addition of perchloric acid to a final concentration of 2% (v/v) and subsequently centrifuged for 2 min at 11000 × *g*.

The resulting supernatant, containing peptides, was analysed by HPLC on a C18 reverse phase analytical column, using a 16–56% (v/v) acetonitrile/water gradient for 50 min.

First order rate constants of the disappearance of peptides were calculated according to Eq. 1.

$$S_t = S_0\, e^{-kt}$$

where S_t = integrated area of non-degraded peptide peak, at time t; S_0 = integrated area of initial peptide peak at $t = 0$; k = first order rate constant of the degradation of the peptide.

2.5. Electrophysiological studies

Female Sprague-Dawley rats weighing 200–250 g (ALAB, Sweden) were initially anaesthetised with methohexital (Brietal, Lilly, Indianapolis, USA, 70 mg/kg, i.p.), decerebrated by aspiration of the forebrain and midbrain and then ventilated. The spinal cord was exposed by laminectomy at the mid thoracic level and sectioned at Th 8–9. An i.t. catheter (PE 10) was implanted caudally to the transection with its tip on the lumbar spinal cord (L4–5). The flexor reflex was elicited by supramaximal test stimuli to the sural nerve innervation area in the left foot with electric shocks (0.5 ms, 10 mA, 1/min) of sufficient strength to activate C-fiber afferents. The flexor reflex was recorded as E.M.G. activity via stainless steel needle electrodes inserted into the ipsilateral posterior biceps femoris/semitendinosus muscles. The number of action potentials elicited during the reflex was integrated over 2 s. Galanin, galanin-(−7–29) and galanin-(−9–29) were dissolved in saline and injected i.t. in a volume of 10 μl followed by 10 μl saline to flush the catheter.

3. Results

The specific binding of ^{125}I-galanin (0.1 nM) to membranes from lumbar dorsal spinal cord could be fully displaced by the two *N*-terminally extended galanin analogues, galanin-(−7–29) and galanin-(−9–29) in the concentration range 10^{-11} to 10^{-4} M with IC_{50} values of 0.13 and 0.14 μM, respectively, which are affinities approximately 100-fold lower than that of galanin-(1–29) with an $IC_{50} \approx 1$ nM. Hill coefficients of the displacement curves for galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) were all unity. Fig. 1, Table 1.

To determine whether galanin-(−7–29) and galanin-(−9–29) act as precursors for a proteolytic formation of galanin-(1–29), which is a high affinity ligand at the spinal galanin receptor or they are intrinsic ligands with low affinity, displacement studies in the absence or presence of a protease inhibitor cocktail (see Methods for composition) were performed. No difference in the displacement of ^{125}I-galanin binding by galanin-(−7–29) or galanin-(−9–29) was found, in the presence of the protease inhibitor cocktail, suggesting that galanin-(−7–29) and galanin-(−9–29) on their own are ligands at the spinal galanin receptor (data not shown).

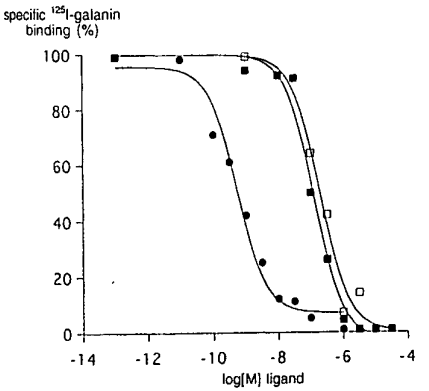

Fig. 1. Displacement of specific ^{125}I-galanin (0.2 nM) binding to membranes (0.2 mg/ml) prepared from lumbar dorsal spinal cord by galanin-(1–29) (filled circles), galanin-(−7–29) (filled squares) and galanin-(−9–29) (open squares) at 37°C for 30 min, with increasing concentrations of displacing ligand. Specific binding was defined as that displaceable by 1 μM galanin. Three independent experiments were performed and each point was determined in triplicate.

Table 1
Affinities and half-lives of galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) in membranes from lumbar dorsal spinal cord

Ligand	Galanin-(1–29)	Galanin-(−7–29)	Galanin-(−9–29)
IC_{50} [a] (nM)	1 ± 0.4	130 ± 60	140 ± 40
$t_{1/2}$ [b] (min)	117 ± 17	271 ± 23	185 ± 19

[a] IC_{50} values are derived from the observed IC_{50} values ($IC_{50(obs)}$) values according to Cheng et al. (1973). $IC_{50} = IC_{50(obs)}/(1 + ([^{125}I\text{-galanin}]/K_d \text{ galanin}))$. [b] Half-lives for galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) (20 μM), when incubated in P_2 membranes from lumbar dorsal spinal cord (0.2 mg/ml), Hepes 5 mM, pH 7.4 at 37°C.

To further confirm that galanin-(−7–29) and galanin-(−9–29) are ligands on their own, and that proteolytic degradation is not required for displacement of ^{125}I-galanin binding, the formation of galanin-(1–29) as a possible peptidolytic product of galanin-(−7–29) and galanin-(−9–29) was examined. Following the degradation of galanin-(−7–29) and galanin-(−9–29) (20 μg/120 μl) after 30-min incubation with membranes from lumbar dorsal spinal cord (0.2 mg/ml), by HPLC analysis, using synthetic porcine galanin-(1–29) as standard, it was shown that no detectable amount, i.e. no or less than 0.5% of the *N*-terminally extended galanin analogues was processed to yield galanin-(1–29) (Fig. 2).

In order to compare the proteolytic stability of these ligands galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) (20 μM) were incubated in membranes (0.2 mg/ml) from lumbar dorsal spinal cord at 0–210 min and their half-lives were determined (Table 1).

The half-lives for galanin-(1–29), galanin-(−7–29) and galanin-(−9–29) were 117 ± 17, 271 ± 23 and 185 ± 19 min, respectively. Thus the half-lives for galanin-(−7–29) and galanin-(−9–29) were significantly longer than for galanin-(1–29). After 30-min incubation in membranes from lumbar dorsal spinal cord, the concentration of remaining ligand, calculated with rate constants obtained from the degradation experiments, was 85, 92 and 90% of the starting concentration, respectively, for galanin-(1–29), galanin-(−7–29) and galanin-(−9–29).

The in vivo experiments showed that i.t. galanin-(−7–29) and galanin-(−9–29) had only very weak

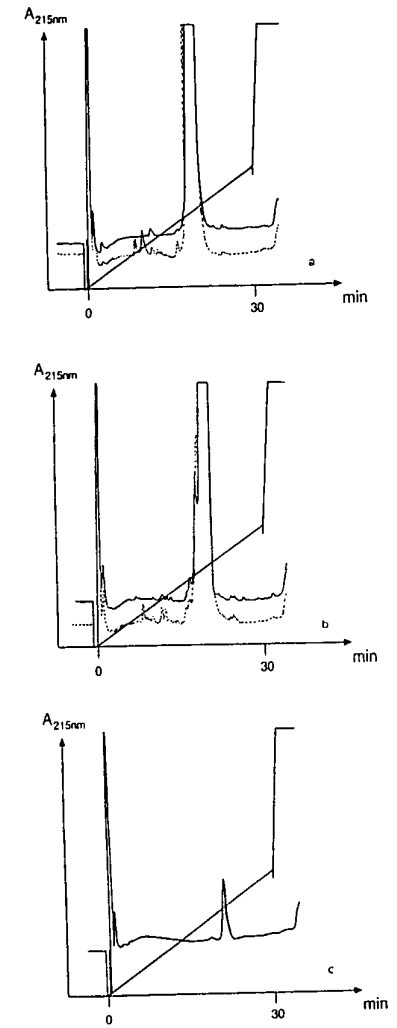

Fig. 2. HPLC profiles of the degradation patterns of (a) galanin-(−7–29) (20 μg/120 μl) and (b) galanin-(−9–29) (20 μg/120 μl) after 0 and 30 min incubation in P_2 membranes from lumbar dorsal spinal cord (0.2 mg/ml). Galanin-(1–29) (0.1 μg) was used as standard. Degradation was stopped and proteins were precipitated by addition of perchloric acid, followed by centrifugation. The resulting supernatant was analysed by HPLC on a C_{18} reverse phase analytical column, using a 16–56% (v/v) acetonitrile/water gradient for 50 min. (a) Solid line: 12.5 μg galanin-(−7–29) after 0 min incubation. Stippled line: 12.5 μg galanin-(−7–29) after 30 min incubation. (b) Solid line: 12.5 μg galanin-(−9–29) after 0 min incubation. Stippled line: 12.5 μg galanin-(−9–29) after 30 min incubation. (c) Solid line: 0.1 μg galanin-(1–29), used as standard.

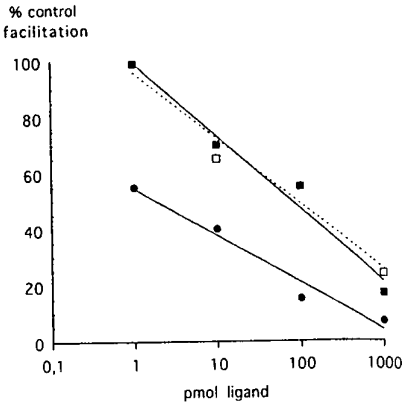

Fig. 3. Antagonistic effect of i.t. galanin-(1–29) (filled circles), galanin-(−7–29) (filled squares) and galanin-(−9–29) (open squares) on the facilitation of the flexor reflex induced by a train of 20 electric shocks at 0.9 Hz which activated C-fibers. The data were collected from 4–13 experiments for each compound at each dose. The regression line for galanin-(1–29) was $y = 16.99x + 62.91$ ($r = 0.603$), for galanin-(−7–29) $y = 26.9x + 113.21$ ($r = 0.82$) and for galanin-(−9–29) $y = 24..29x + 111.53$ ($r = 0.86$). Analysis of variance indicated that all regressions were significant ($P < 0.01$). The dose required for 50% antagonism of C-fiber conditioning stimulus-induced reflex facilitation was 5.6 pmol for galanin-(1–29), 324 pmol for galanin-(−7–29) and 339 pmol for galanin-(−9–29).

effects on spinal cord reflex excitability compared to galanin (1–29). At doses of 3–300 pmol, when galanin-(1–29) has a pure facilitatory effect, the extended peptides had no effect. Consistent reflex facilitation by galanin-(−7–29) and galanin-(−9–29) was only observed at a dose of 3 nmol.

The characteristic inhibitory effect of galanin-(1–29) on the facilitation of the flexor reflex induced by conditioning stimulation of C-fiber afferents was mimicked by both galanin-(−7–29) and galanin-(−9–29), although the potencies of these peptides (ED_{50}, 324 and 339 pmol, respectively), were lower than that of galanin-(1–29) (ED_{50}, 5.6 pmol) (Fig. 3).

4. Discussion

The present study showed that the two endogenously occurring *N*-terminally extended forms of galanin, galanin-(−7–29) and galanin-(−9–29), corresponding to those recently isolated from porcine adrenal medulla (Bersani et al., 1991b), recognize galanin receptors with affinities of ≈ 0.1 μM, and behave as agonists at spinal galanin receptors. Ligand binding studies suggested the presence of a single population of G-protein-coupled receptors for galanin-(1–29) with $IC_{50} \approx 1$ nM and $n_H = 1$ in the rat spinal

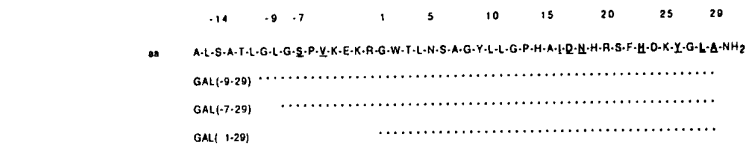

Fig. 4. Amino acid sequence of porcine galanin-(1–29), galanin-(−7–29) and galanin-(−9–29). Bold and underlined amino acids indicate non consensus residues through six different species, including porcine, rat, bovine, chicken, sheep and human sequences.

cord (Bedecs et al., 1992). Previous structure-activity relationship studies showed that the *N*-terminal part of galanin is of importance, and that the amino acids Gly^1, Trp^2, Asn^5, Leu^{10} and Leu^{11} are the main pharmacophores (Land et al., 1991b, see Fig. 4 for sequences). *N*-Acetylation of Gly^1 in galanin-(1–16) resulted in a > 100-fold decrease of the affinity, indicating the importance of a free *N*-terminal. L-Ile or L-Ala substitution of L-Trp^2 in galanin-(1–29) (Lagny-Pourmir et al., 1989) and galanin-(1–16) (Land et al., 1991b), respectively rendered these molecules completely inactive at the central (brain and hypothalamic) galanin receptor. The decreased affinities of galanin-(−7–29) and galanin-(−9–29) as compared to that of galanin-(1–29) are not surprising, since the free *N*-terminal Gly^1 is no longer present and the accessibility of the optimal conformation will be limited in these *N*-terminally extended galanin peptides.

The characteristic depressive effect of galanin on C-fiber-mediated spinal cord sensitisation is dose-related (Xu et al., 1990) and analysis of the effect of galanin-(−7–29) and galanin-(−9–29) for this parameter indicated that both of these peptides exhibited weaker potencies for activating the galanin receptor than did galanin-(1–29). This finding of lower efficacy is consistent with the binding data showing lower affinities for galanin-(−7–29) and galanin-(−9–29) than for galanin-(1–29). The half-lives of galanin-(−7–29) and galanin-(−9–29) were significantly longer than that of galanin-(1–29), in accordance with earlier findings that galanin-(1–29) is degraded at the *N*-terminus by dipeptidyl aminopeptidases and other peptidases (Land et al., 1991a) (Bedecs et al. in preparation). We have at present no explanation for the significantly longer half-life of galanin-(−7–29) than that of galanin-(−9–29). Biophysical studies will be initiated to examine how the amino acids, L^{-9} G^{-8}, shorten the half-life of galanin-(−9–29). It should also be noted that even though galanin-(−7–29) has a significantly longer half-life than galanin-(−9–29), their IC_{50} and ED_{50} values are almost identical – again suggesting that it is not a degradation product that acts at the spinal galanin receptor. In summary we found that the *N*-terminally extended forms of galanin are proteolytically more stable than galanin and that no detectable amount of galanin-(1–29) is formed from them, thus they are not bona fide precursors of galanin-(1–29), but are intrinsic ligands, with low agonist activity, in the rat spinal cord. Whether or not galanin-(−7–29) and galanin-(−9–29) can act as precursors of galanin-(1–29) in the porcine adrenal medulla cannot be concluded from this study with the rat spinal cord, although the precursor sequences of rat and porcine galanin show a high degree of homology at the *N*-terminal extension.

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Dihydropyridine ligands influence the evoked release of oxytocin and vasopressin dependent on stimulation conditions

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Abstract

The effects of dihydropyridine ligands on the electrically evoked release of neurohypophysial hormones from isolated, rat neurointermediate lobes were investigated as a function of all combinations of two pulse widths (0.2 and 2 ms) and three stimulation frequencies (6.5, 13 and 30 Hz). The dihydropyridine agonist (*S*)-(+)-202–791 potentiated concentration dependently the release of both oxytocin and vasopressin at a pulse width of 2 ms and a frequency of 6.5 Hz. This effect of (*S*)-(+)-202–791 was abolished by the antagonist (−)-nitrendipine and stereospecifically by (*R*)-(−)-202–791 (only vasopressin). The antagonist (*R*)-(−)-202–791 alone inhibited the release of oxytocin at 13 Hz and 2 ms. The results presented show that the effects of the dihydropyridine ligands are dependent on the stimulation conditions, and thus demonstrate that the entry of Ca^{2+} through the dihydropyridine sensitive L-type Ca^{2+} channel is associated with electrically evoked release of neurohypophysial hormones under certain conditions.

Key words: Oxytocin; Vasopressin; Ca^{2+} channel, L-type; Dihydropyridine; Neurointermediate lobe; (Electrical stimulation)

1. Introduction

The release of hormones from the neurohypophysis is initiated by action potentials propagated from the magnocellular cell bodies. Depolarization of the nerve terminals promotes entry of Ca^{2+} from the extracellular environment (Brethes et al., 1987; Shibuki, 1990; Stuenkel, 1990; Fatatis et al., 1992), through voltage-activated Ca^{2+} channels (Cazalis et al., 1987; Dayanithi et al., 1988; Obaid et al., 1989; Von Spreckelsen et al., 1990; Stuenkel, 1991; Kato et al., 1992). This rise in intraterminal free Ca^{2+} triggers exocytose from neurosecretory nerve endings (Lim et al., 1990; Lindau et al., 1992).

Evidence is available describing multiple types of voltage-activated Ca^{2+} channels which differ in molecular, electrophysiological and pharmacological properties. The major classes of voltage-activated Ca^{2+} channels are known as T, N, L and P (Tsien et al., 1988; Tsien, 1990; Scott et al., 1991).

In isolated terminals from the neurohypophysis two types of high voltage-activated Ca^{2+} channels have been characterized using patch-clamp techniques. One of these corresponds to the dihydropyridine-sensitive L-type channel, while the other is a dihydropyridine-insensitive channel of the N-type family (Lemos and Nowycky, 1989, 1991).

The peptide toxin ω-conotoxin GVIA has been shown to block both N- and L-type Ca^{2+} currents in neurohypophysial nerve terminals (Wang et al., 1992) and to inhibit high K^+- as well as electrically evoked release of vasopressin from isolated neurohypophysis (Dayanithi et al., 1988; Von Spreckelsen et al., 1990). The influence of the dihydropyridines seems to be more complex. During high K^+-induced depolarizations the dihydropyridine agonist Bay K 8644 [(*R,S*)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-trifluoromethylphenyl)-3-pyridinecarboxylic acid methyl ester] was shown to potentiate the release of vasopressin (Cazalis et al., 1987), while the dihydropyridine antagonists nicardipine, nitrendipine and nimodipine inhibited the peptide release (Cazalis et al., 1987; Dayanithi et al., 1988; Fatatis et al., 1992; Kato et al., 1992). On the

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